VITRICATIONTION OF MONONUCLEAR CELLS ISOLATED FROM HUMAN UMBILICAL CORD BLOOD

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CERTIFICATE

This is to certify that the project report entitle "**Vitrification of mononuclear cells isolated from umbilical cord blood**" submitted by **MARK LALDUHSAKA** (108BM020) in the partial fulfilment of the requirement for the degree of the B.Tech in Biomedical Engineering, National Institute of Technology, Rourkela is an authentic work carried out by him under my supervision. To the best of my knowledge the matter embodied in the report has not been submitted to any other Institute/University for any degree.

Date: 9th May 2012

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ABSTRACT

Mononuclear cells present in the blood are responsible for producing the different components of the blood as they contain hematopoietic stem cells. They are also found to contain mesenchymal stem cells which can be differentiated into cartilage, connective tissue and bone tissue. For these reasons, steps had been taken to preserve them for use in tissue engineering. Three different techniques of cryopreservation had been carried out for MNCs isolated from umbilical cord blood and the viability of each was compared. Here, we introduced a new set up for vitrifying cells which proved to be a scalable cryopreserving tool. In this set up N_2 vapour was used for dispersing the droplets of MNC solution supplied by a pump into the liquid nitrogen. N_2 not only dispersed the droplets but also pre cooled the dispersed droplets before it reached liquid nitrogen. The MNC droplets were collected by cell strainers kept in liquid nitrogen it was stored in vapour phase liquid nitrogen ($-150^{o}C$) after vitrification, which was later thawed and tested for viability.

1. INTRODUCTION

Cryogenics is that branch in physics that deals with the production of very low temperatures and the study of its effect on matter. The temperatures involved with cryogenics should be lower than -150°C (-238°F or 120°K) [1]. Cryobiology is the study of different effects of low temperature on biological system [1]. Cryopreservation is an aspect of cryobiology that deals with different process of storing and preserving biological material by cooling it down to cryogenic temperatures [1]. It had been successfully cryopreserved different biological material such as Red blood cells, embryo, stem cells, etc. [2,3,4]. In this way, cryopreservation will provide continuous source of genetically stable living cells and tissues for a variety of purposes, including research and biomedical processes in the near future.

According to cryobiology, at these low temperatures, all biological activities are stopped. This includes the biochemical reaction that may lead to cell death [1]. However, most of the cell is made up of water so cells may undergo damage due to freezing i.e., damage due to ice formation in the extracellular and intracellular level or damage due to the change in phase when approaching room temperature [5,6,7]. In order to prevent this damage, an anti-freeze compounds known as cryoprotectant agents (CPA) are used to protect the cells or tissues by avoiding or decreasing ice crystal formation [8]. Some of the conventional CPAs are glycerol, ethylene glycol (EG) and dimethyl sulfoxide (DMSO). However, cryoprotectants can be toxic in different ways, and different studies showed the different toxicity of CPAs for different types of cells which will be discuss later.

1.1. Different techniques used for cryopreservation: There are different techniques used in cryopreservation. Slow freezing and rapid cooling are the main classification. In slow freezing the cooling rate are controlled, usually below 10^{o}C/min [9] or kept at constant rate of 1^{o}C with the help of some freezing container like “Mr. Frosty” [10]. Different experiment had been performed for cells with the slow freezing process [11]. Although this method was considered effective, cryoinjury still occurs as a result of cell shrinkage during cooling and warming [12,13], toxicity due to increasing solutes concentration [14,15,16] and intracellular ice formation (IIF) [17]. In contrast, vitrification as a method of cryopreservation has

provided a means to reduce these cryoinjuries significantly [18,19]. The use of N_2 gas to for developing micro-droplets was also introduced [2]. This shows significant result over classic straw plunging technique [20] of vitrification.

1.2. Umbilical cord blood: Human umbilical cord blood proved to be a rich source of multipotent stem cells for clinical application in neurodegenerative diseases [23,24]. Umbilical cord blood is obtained from the umbilical cord at the time of child birth [21]. The plancenta is one of the best sources of stem cells, and as the cord blood is coming from the placenta, the cord blood itself is a good source of stem cells [22]. Mononuclear cells (MNCs) derived from cord blood mainly comprises of a heterogeneous population of hematopoietic stem cells and mesenchymal stem cells [25,26]. This is why MNCs are used in therapy for degenerative conditions [25].

1.3. Objective

- To create a set up for scalable vitrification method for cells suspension and to compare its viability with the normal straw plunging method.

2. LITERATURE REVIEW

The main objective of cryopreservation is to increase the viability of cells for each technique and each CPA used. Viability rate simply defines the quality of preservation. But viability of cells greatly affected by the rate of cooling a well as the toxicity of CPA. Conventional CPAs such as glycerol, EG and DMSO may cause different effects on the physiology of human being [27]. But these are the long term problems if we relate this to cryoprotectant toxicity in cryobiology. A study of mouse blastocysts using six CPAs in 30% (v/v) shows EG to be least toxic than DMSO and glycerol [28]. However, for oyster embryos, DMSO is less toxic than EG [29]. In different studies, appropriate mixture of these CPA shows significant result. The most toxic compound, formamide, when combined with DMSO shows the least toxicity as CPA for kidney slices [30]. DMSO considerably reduces formamide toxicity (and apparently vice-versa). So, the main aim of cryobiologists is to create a good mixture of CPA with low toxicity, low viscosity and good vitrifying capability.

It is found that unlike polyols (glycerol, EG etc), DMSO toxicity can be reduced by mixing it with other CPAs [31].

Toxicity decrease as:

Formamide > Ethylene glycol > Propylene glycol > N-methyl formamide

In the year 1993, mixture of EG and DMSO is used for the vitrification of bovine embryos [32]. The mixture show significant result. The similar mixture of CPA was again used for the vitrification of blastocyst in the year 2000 [33]. Here the same mixture with a bit of change in concentration is used.

3. MATERIALS AND METHODS

Different protocols and methods followed for all the steps involving in the cryopreservation on mononuclear cells are:

3.1. Isolation of mononuclear cells from cord blood

Collection of blood sample

Cord blood is collected from blood bag under sterile conditions.

Isolation of mononuclear cells

1. The bio-safety cabinet is swap with 70% ethanol.
2. The unprocessed cord blood is diluted in the ratio 1:1 with phosphate buffered saline (PBS).
3. Carefully, 30 ml of diluted cells are layered on 15 ml of Ficoll-Hypaque (1.077 g/ml density) in each 50-ml conical tube without disturbing the Ficoll-Hypaque interface.
4. It was centrifuged for 20-30 min at 450g at room temperature with the brake set to the "off" position.
5. After centrifugation, a layer of mononuclear cells should be visible on top of the Ficoll-Hypaque phase, as they have lower density than Ficoll-Hypaque solution as shown in Fig. 1.
6. Carefully the mononuclear cellswas harvested from the buffy layer located at the interface between the medium and Ficoll using 3-ml plastic Pasteur pipette.
7. The cells were washed with PBS and recover the cells by centrifugation for 10-15 min at 300g and room temperature.
8. The supernatant was discarded, the cell pellet was re-suspended and the cells were counted using a haemocytometer.

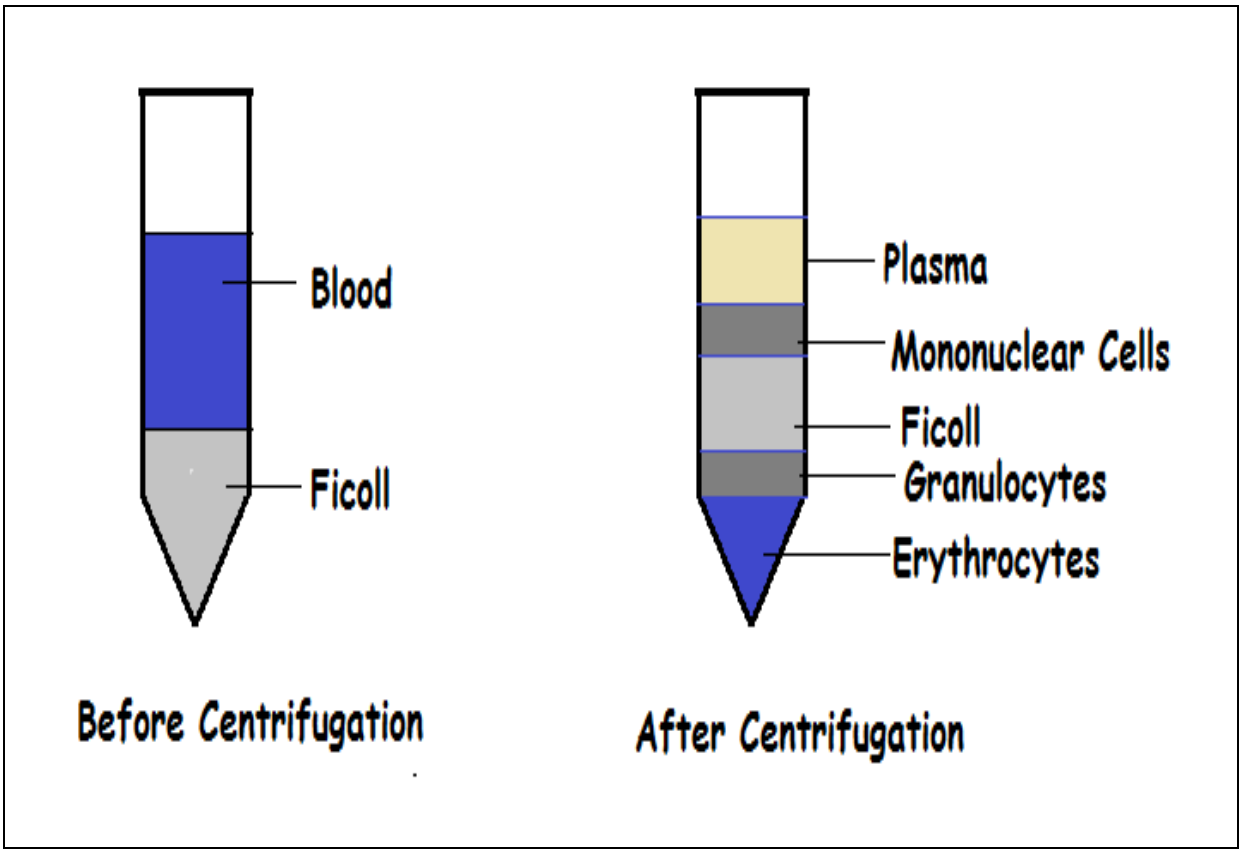


Figure1: Density gradient centrifugation

3.2. Experiment No.1

Cryopreservation using -80°C deep freezer

Cryoprotectant solution composition: 10% DMSO
15% FBS in DMEM Media
Add cell suspension such that the final concentration of DMSO is 10% (v/v)

PROCEDURE

1. Prepare fresh cryoprotectant solution and store at 2°C to 8°C until use
2. Simultaneously take the Mr. Frosty" freezing container and pour isopropanol in it (upto 60% of the height of the wall) and keep it in -6°C to -8 °C freezer
3. Take the cell suspension isolated from processed peripheral blood or cord blood (refer supplement) and determine the total number of cells using hemacytometer, cell

counter and Trypan Blue exclusion. Cell count should not be less than 1x 10^7 viable cells /ml

4. Centrifuge the cell suspension at approximately 100–200 *g* for 5 - 10 minutes
5. After centrifugation decant the supernatant without disturbing the cell pellet
6. To the pellet add pre-cooled cryoprotectant solution, FBS and basal media as per requirement
7. Place 1.0 ml of cell mixture in each cryovial
8. Lable the cryovials
9. Rapidly transfer the vials to a precooled (4°C) Nalgene freezing container (containing isopropanol), and place immediately in a freezer at –80°C
10. Freezing is generally carried out overnight
11. After freezing for appropriate time, remove " Mr. Frosty" freezing from the -80 °C deep freezer and take out the vials containing the frozen cells
12. Now thaw the cells as per protocol mentioned in 3.4.
13. Perform cell viability analysis according to protocol in 3.5.

3.3. Experiment No.2

Vitrification by plunging straw containing the solution into Liquid Nitrogen

Cryoprotectant solution composition: 10% DMSO

10% FBS in DMEM Media

20% EG

1M sacrose

Add cell suspension such that the final concentration of DMSO is 10% (v/v)

PROCEDURE

Cell Preparation for vitrification

1. Prepare fresh vitrification solution, filter it using Syringe filter and store at 2°C to 8°C until use.

2. Take the cell suspension isolated from processed peripheral blood or cord blood(refer supplement) and determine the total number of cells using haemocytometer, cell counter and Trypan Blue exclusion. Cell count should not be less than 1x 107 viable cells /ml.
3. Centrifuge the cell suspension at approximately 100–200 g for 5 - 10 minutes.
4. Decant the supernatant without disturbing the cell pellet.
5. To the cell pellet add vitrification solution.
6. Allow MNCs to sit in freezing medium (hypertonic vitrification solution) until they reach osmotic equilibrium.

Loading MNCs into Plastic Straw

1. Using 1ml syringe load the vitrification solution containing MNC's into the plastic straw.
2. Label the straw using printed labels attached to either a straw sealing plug or to another straw connected with a straw adapter.
3. Remove the straw from the vitrification solution containing MNC's, and aspirate approximately 0.15 cm of air to create a tiny air bubble within the straw.
4. Aspirate a single MNC in approximately 0.8 cm of hypertonic vitrification solution into the straw.
5. Remove the straw from the vitrification solution and aspirate approximately 0.15cm of air to create a second tiny air bubble within the straw (air bubbles serve to physically isolate the MNC within the straw).
6. Aspirate approximately 9.1 cm of vitrification solution to completely fill the straw, being certain to draw in a sufficient volume of vitrification solution to moisten the polyvinylchloride (PVC) powder that exists within the cotton plug end of the straw.
7. The vitrification solution causes the PVC powder to gel and seal that end of the straw.
8. Seal the other end of the straw with PVC powder, plastic straw sealing plugs, or a heat sealer.
9. Place the straws loaded with vitrification solution and MNC's into the control rate freezer whose temperature has been cooled from ambient temperature to -6°C
10. Allow the sample to sit at this temperature for at least 2 minutes before proceeding

Seeding of the sample

1. Once the MNC's have cooled to -6°C, use a pair of tongs supercooled in liquid nitrogen (or a cotton-tipped stick immersed in liquid nitrogen) to induce ice crystal formation in the vitrification solution inside the straw by touching the tongs (or cotton tipped stick) to the column of solution.
2. The water in the vitrification solution will crystallize in the region exposed to liquid nitrogen, and ice crystals will spread to the column of vitrification solution immediately surrounding the MNC's.
3. Hold MNC's at seeding temperature for 10 minutes before further cooling.
4. Cool the MNC's at a rate of 0.5°C/min down to a temperature of -34°C. This cooling rate is important to ensure continued dehydration of the MNC's
5. Hold the MNC's at -34°C for 10 minutes before plunging MNC's into liquid nitrogen (-196°C).
6. Place cryopreserved MNC's into an appropriately labelled goblet (filled with liquid nitrogen) attached to an appropriately labelled cane, and place the cane into a canister of a liquid nitrogen dewar for short- or long-term storage

After completing the above procedure, thawing is done using the procedure in 3.4 and cells are counted to check the viability.

3.4. Thawing and cell recovery

After the cells are stored in liquid nitrogen for a night, the cells are thawed and recovered by the following procedure:

1. Carefully remove the MNCs (or mononuclear cells) vial from Liquid Nitrogen Storage Tank.
2. Rapidly thaw frozen vial of cells in a 37°C water bath, until just a small frozen chunk remains in the vial.
3. Spray vial with 70% ethanol to decontaminate it and transfer it to the sterile tissue culture hood.
4. Aseptically transfer the entire contents of the vial into a 15 ml conical tube using a 5 ml pipette.
5. Rinse the vial with 1 ml of thawing media and transfer it to 15 ml centrifuge tube.

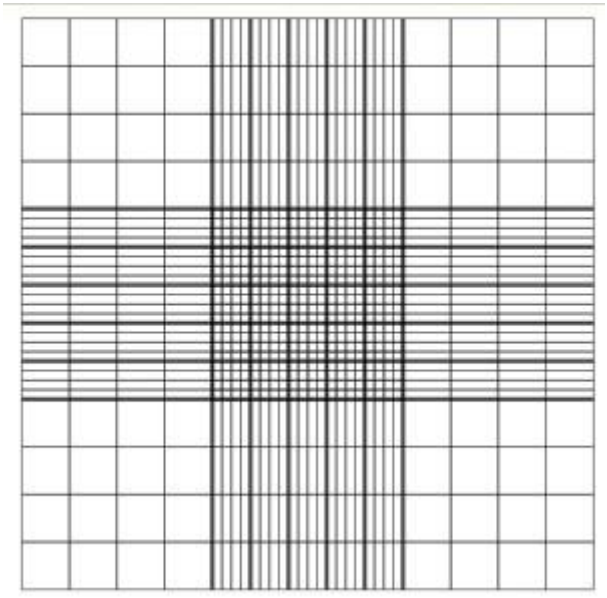
6. Slowly, add 4 ml of thawing media drop-wise to cells in the 15ml conical tube. While adding the medium, gently move the tube back and forth to mix the cells. (This reduces osmotic shock to the cells).
7. Centrifuge the 15 ml tube with the cell suspension at 200*g* for 2 minutes at room temperature.
8. Carefully aspirate and discard the supernatant without disturbing the cell pellet.
9. Re-suspend the cell pellet in pre-warmed thawing media (e.g. 4ml/60 mm dish) using a 5 ml pipette and gently pipette the cells up and down until cell pellet is fully dispersed.
10. Now check the cell viability using trypan blue and haemocytometer following the protocol mentioned in page no. 15
11. A viability of greater than 70% is expected, however if the viability is less than 70%, it may take longer for the cells to reach confluence.
12. Now take sufficient amount of pre warmed DMEM and gently pipette the cells up and down in it.
13. Now transfer the above into a culture dish.

3.5. Cell Viability

To test the viability, cells are counted twice, before cooling and after thawed, and are compared to see the percentage of cells survived. Following is the procedure for counting the number of cells.

1. To prepare the haemocytometer, the mirror-like polished surface is carefully cleaned with lens paper and ethanol
2. The coverslips (which is thicker than those for conventional microscopy) should also be cleaned
3. Take 200 µl of the cell suspension into a 1.5 ml in the centrifuge tube
4. Add 300 µl of PBS and 500 µl of 0.4% trypan blue solution to the cell suspension (creating a dilution factor of 5)
5. Mix thoroughly and allow to stand for 5 to 15 minutes
6. **Note**: Trypan blue dye is taken up by the cell membrane of dead cells only, however if cells are exposed to trypan blue for extended periods of time, viable cells may begin to take up dye as well as non-viable cells, thus, try to do cell counts within half an hour after dye solution is added

7. With a cover-slip in place, use a micro pipette and transfer a small amount of the trypan blue-cell suspension to a chamber on the haemocytometer
8. This is done by carefully touching the edge of the cover-slip with the pipette tip and allowing the chamber to fill by capillary action. Do not overfill or underfill the chambers
9. The counting chamber is then placed on the microscope stage and the counting grid is brought into focus at low power
10. One entire grid on standard haemocytometers with Neubauer rulings can be seen at 50x (5x objective)
11. The main divisions separate the grid into 9 large squares. Each square has a surface area of $1mm^2$, and the depth of the chamber is 0.1mm. Each square of the haemocytometer (with cover slip in place) represents a total volume of 0.1 mm^3or 10^{-4} cm^3. Since 1 cm3 =1 ml, the subsequent cell concentration per ml can be determined.



Cells per ml = the average count per square x the dilution factor x 10^4 (count 10 squares)

12. Count all the cells (non-viable cells stain blue, viable cells will remain opaque) in the 1mm center square and the four corner squares
13. Refer to diagram above. Keep a separate count of viable and non-viable cells. If greater than 25% of cells are non-viable, the culture is not being maintained on the appropriate amount of media; re-incubate culture and adjust the volume of media according to the confluency of the cells and the appearance of the media

14. If there are less than 50 or more than 200 cells per large square, repeat the procedure adjusting to an appropriate dilution factor
15. Repeat the count using the other chamber of the haemocytometer

3.6. Peristaltic pump

For the continuous flow of MNCs and CPA solution, peristaltic pump is used. The flow rate of the cell solution is controlled by the revolutions per minute (rpm) of the peristaltic pump. Different readings of time taken to complete 20mL for different rpm(s) are noted down shown in Table1. According to the table, a graph is plot between time taken and the rpm shown in Figure2. The graph represented a curve of rectangular hyperbola. The simple formula of rectangular hyperbola, xy=c is used to deduce the formula of rpm dependent flow rate.

Sl. No.	**rpm (x)**	**time taken in sec (y)**	**x*y**
1	5	283	1415
2	10	137	1370
3	15	91	1365
4	20	68	1360
5	25	55	1375

Table1: Change of time with rpm

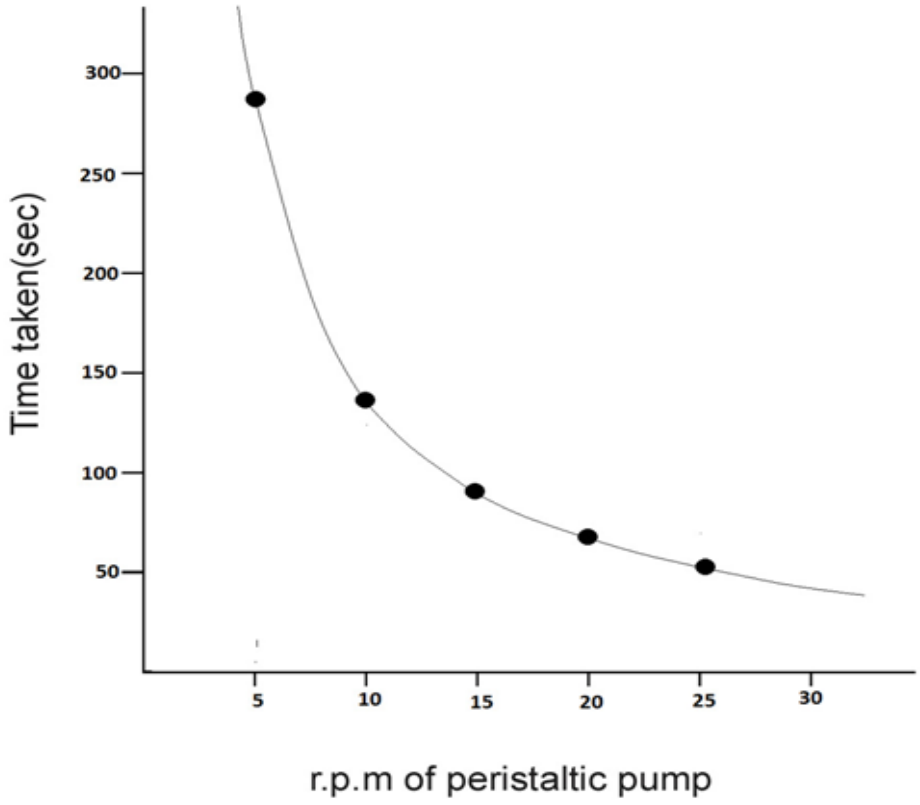


Figure2: Time vs rpm graph for peristaltic pump

From the table we can approximate the value of ‘c’ which is always constant for any values of ‘x’ and ‘y’. By taking the mean of each values in column number 4 (x*y column) we get the approximate value of ‘c’.

$$c = (1415+1370+1365+1360+1375)/5 = 1377 \sim 1370$$

Each of the noted time(s) are for 20 mL, so,

$$\text{Flow rate} = 20/y \text{ in mL/sec}$$

But we need mL/min, so we multiply the whole term by 60, therefore,

$$\text{Flow rate (say z)} = (20*60)/y \text{ in mL/min}$$

Hence,

$$x = c/y = 1370/(1200/z) = (1370*z)/1200$$

Putting the value of z (flow rate required), we can find out the rpm required ‘x’ for the required flow rate.

3.7. Experimental design

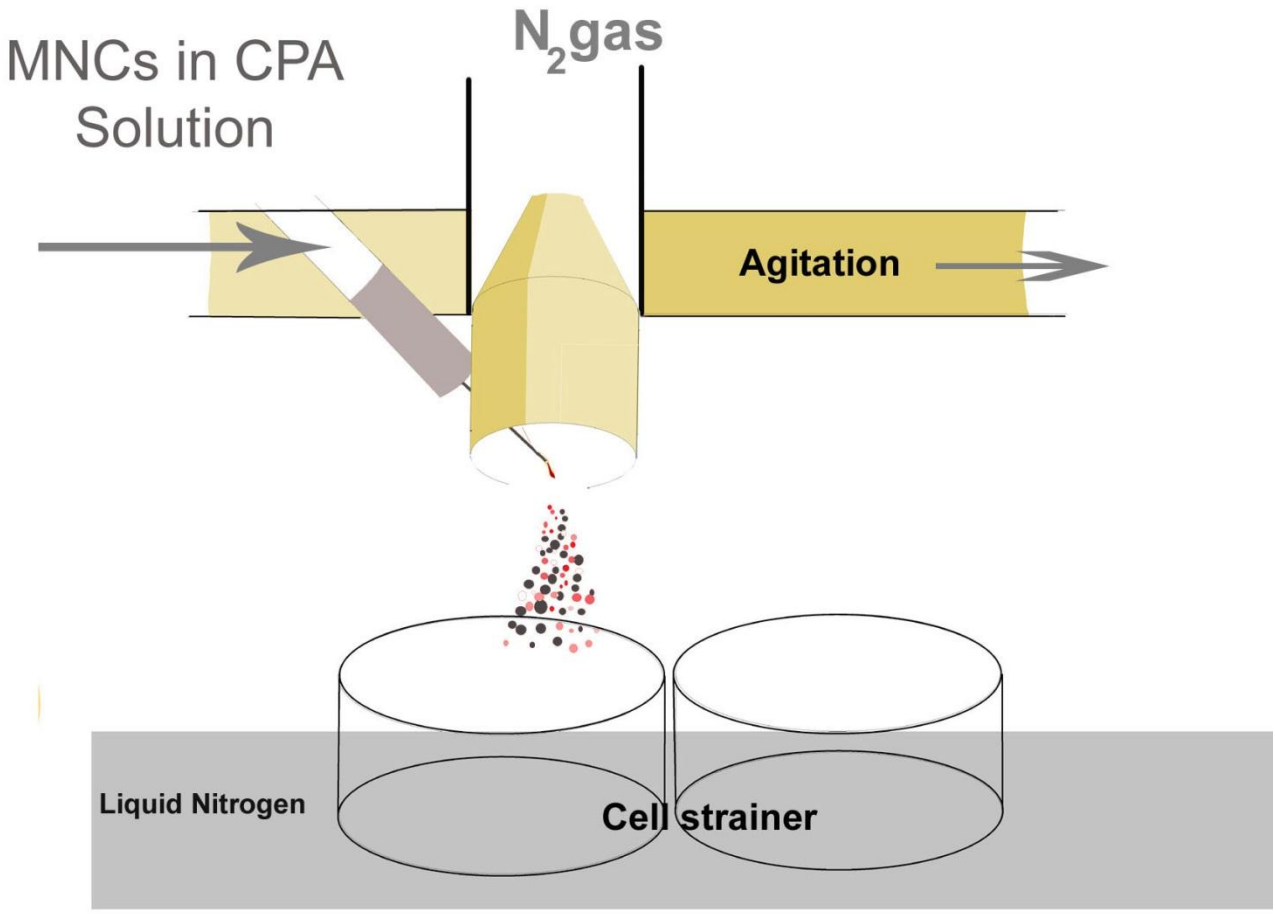


Figure3: Setup design for the new vitrification technique.

A silicon rubber pipe 4.8mm diameter is connected to the peristaltic pump. One end of the pipe is kept inside a beaker containing a sample mixture of CPA and MNCs and the other end is connected with a hypodermic needle, 22G × 1¼" (0.7 × 30mm), where the ejection of sample takes place (Figure4). This needle is inserted through the N_2 vapour outlet, which is a cone shape plastic opening at the base and the top (0.8mm) as shown in Figure2. N_2 vapour flows through a plastic pipe connected to a Liquid Nitrogen tank.

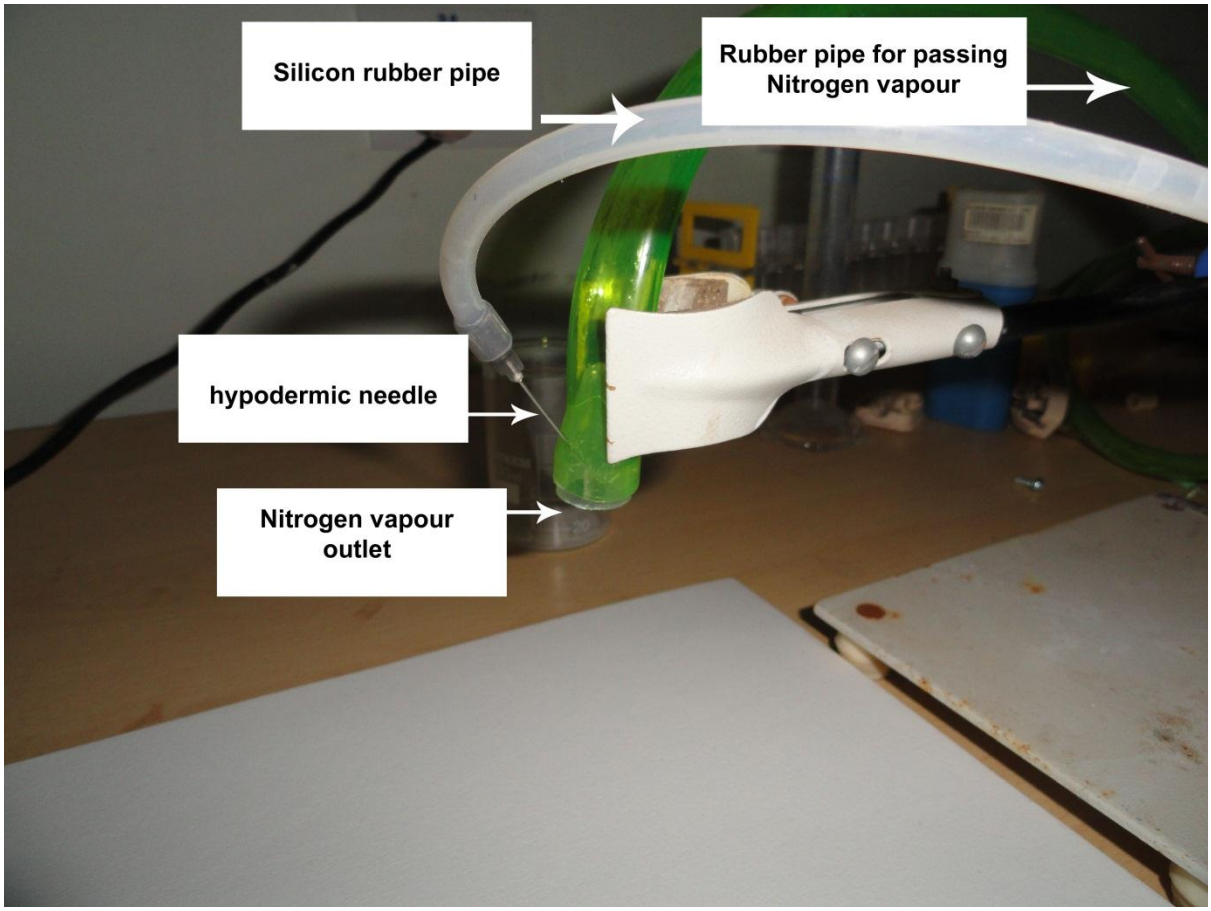


Figure4: N_2 vapour outlet (cone-like plastic) for dispersion of droplets coming from hypodermic needle.

When the pressure developed inside the Liquid Nitrogen tank is 0.5 bar, N_2 gas is released from the outlet of the tank which is passed through a rubber pipe and finally to the outlet (Figure6). The N_2 gas coming out of the outlet dispersed the sample solution that is made to flow using peristaltic pump through the silicon pipe to the orifice opening, shown in Figure6.

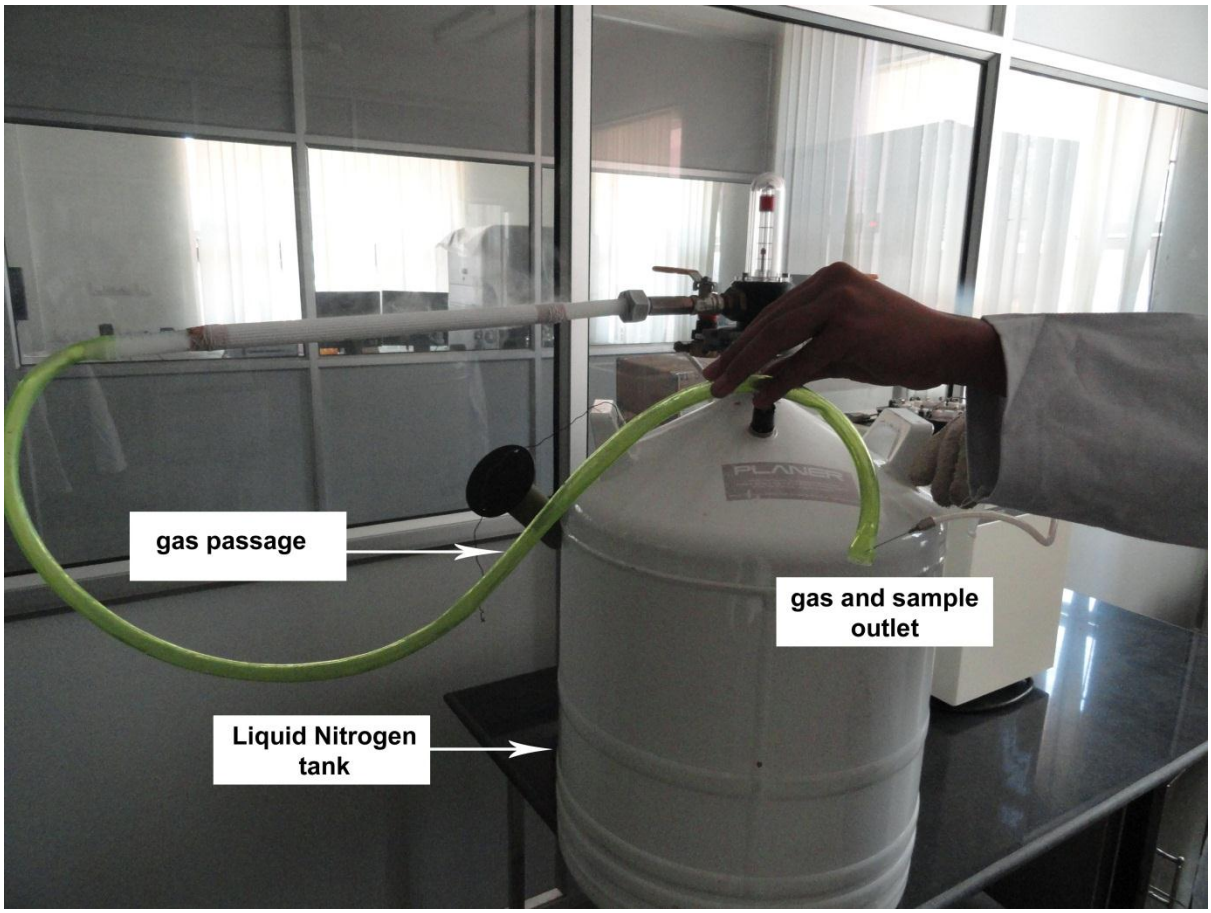


Figure5: N_2 gas passage from Liquid Nitrogen tank

The connector of the gas outlet of liquid nitrogen tank was not available, so, it was self-constructed by aluminium pipe bought from local hardware store. All the connections were sealed by polytetrafluoroethylene (PTFE) also known as thread seal tape (from local hardware store).

4. RESULTS AND DISCUSSION

4.1. Vitrifiction using plunging method

Before vetrifying the sample prepared in 3.3, cells were counted using trypan blue and haemocytometer. The cell count was $1.2x10^7$cells/ml. After vetrification was done following section 3.3 in Experiment no 2, the cell were thawed using the procedure in section 3.4, and the cells were counted as in section 3.5. Viable cells were counted to be around $0.8x10^7$calls/ml. This is approximately around 67% viability.

4.2. Vitrification using N_2 vapour gas

Using the formula given in the section 3.6,

$$x = (1370*z)/1200$$

we varied the different values of flow rate in ml/min (z) and find the necessary rpm to be set on the peristaltic pump. Table2 shows the different rpm used for optimum flow rate.

flow rate in ml/min (z)	**rpm required (x)**
5	5.7
2.5	2.8
0.5	0.6

Table2: flow rate into rpm

This different flow rates were tested. A solution was prepared with 10% DMSO (v/v), 3% trypan blue (v/v) and water that made up rest of the volume. This solution had almost similar density with cells and CPAs solution. The solution prepared was made to disperse over Fabriano handmade-paper (9.5" X 12.5", 300g/m^2), using N_2 vapour maintained at 0.5 bar pressure.

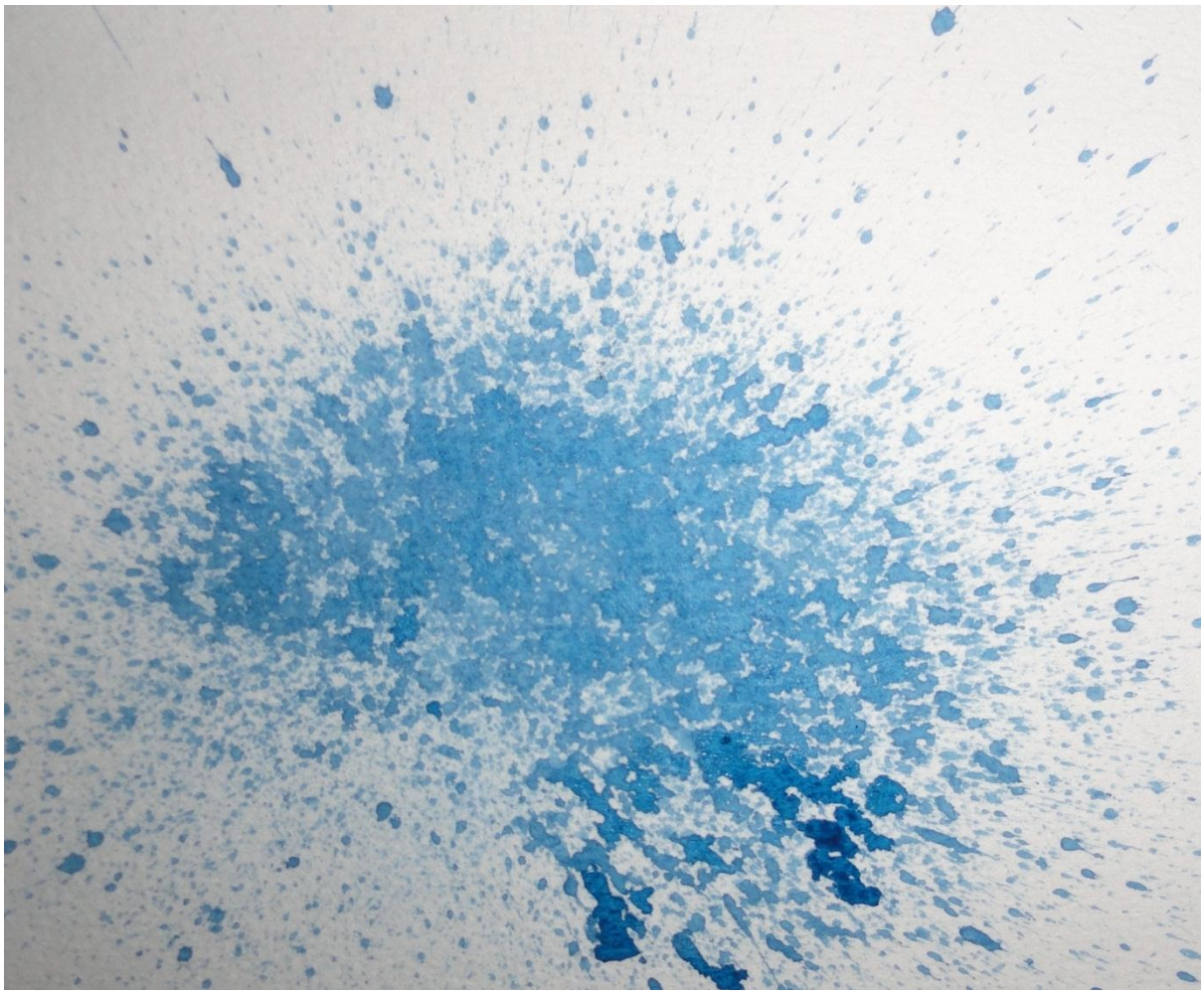


Figure6: Flow rate of 5ml/min.

In Figure6, we can see that droplets forme over the paper are big, and dispersion effect of N_2 gas is not that effective.

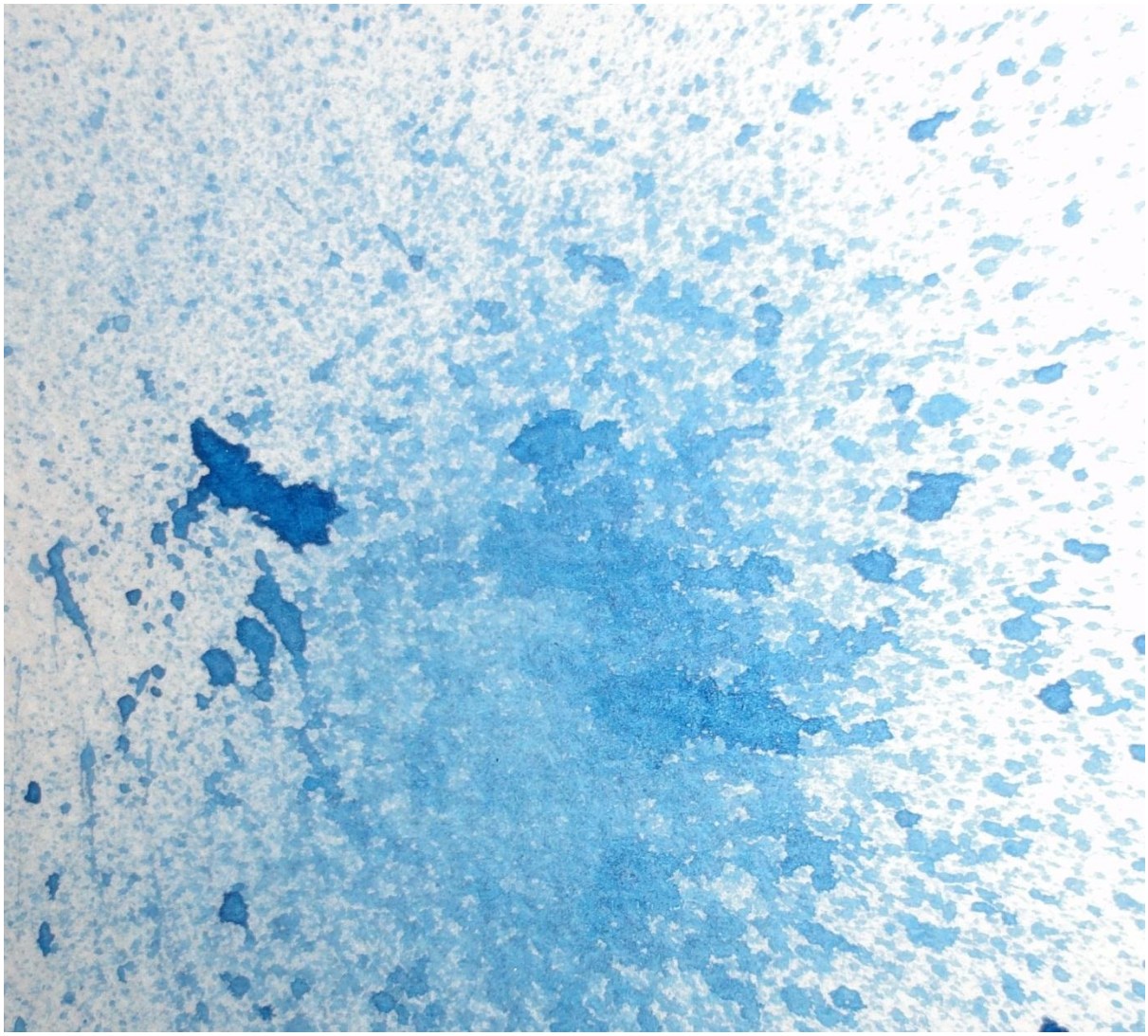


Figure7: Flow rate of 2.5ml/min.

In Figure7, almost the same effect was seen, so the pressure of N_2 gas was increased further to 0.8bar and the flow rate of sample was also decreased to 0.5ml/min.

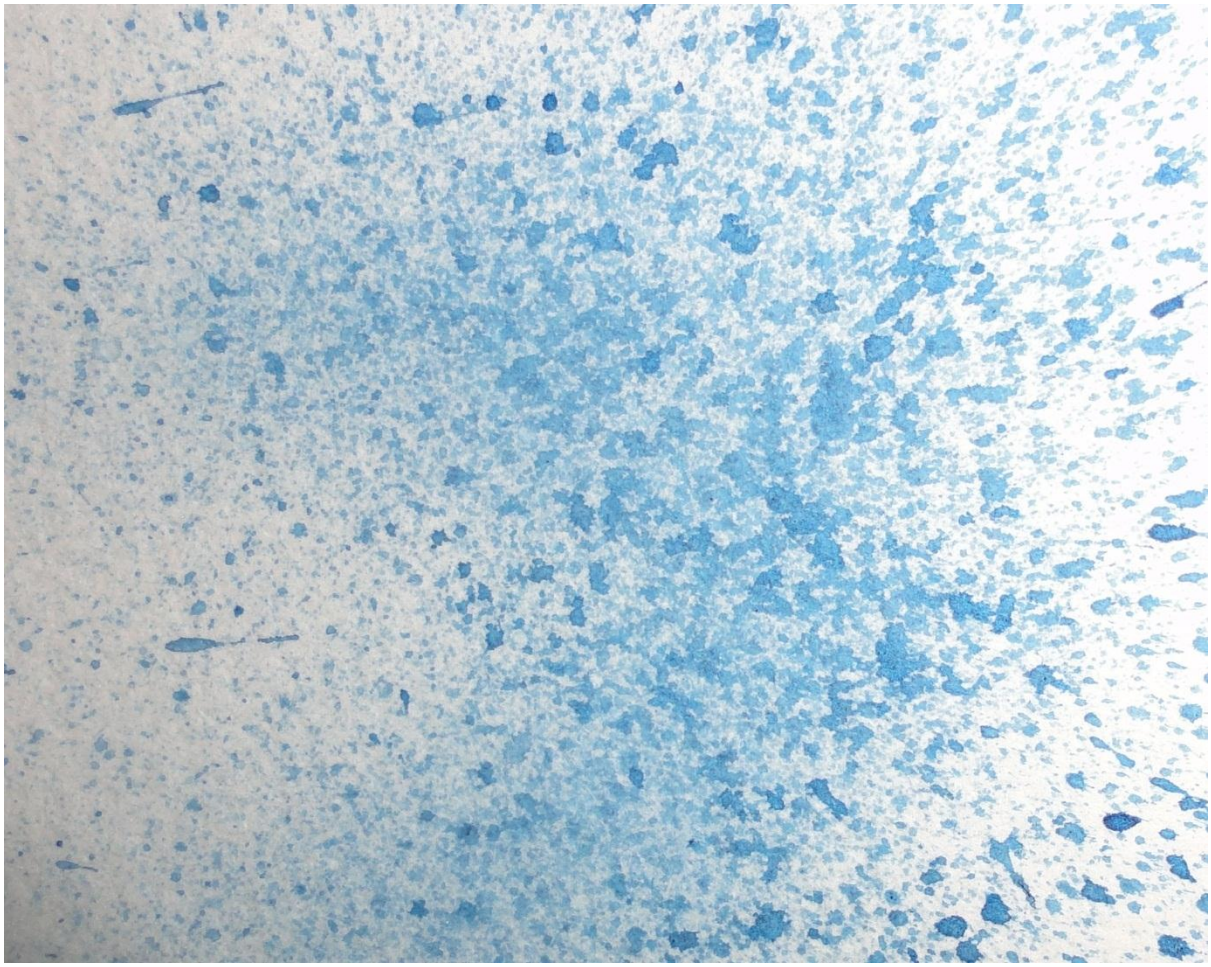


Figure8: Flow rate of 0.5ml/min and 0.8bar pressure for N_2 gas.

After these changes, from Figure8, we can see the effective disperse effect of N_2 vapour gas over the paper. As we cn see, the droplets were evn more dispersed, which results in smaller size of droplets, till 1mm-3mm in diameter (shown in Figure10).

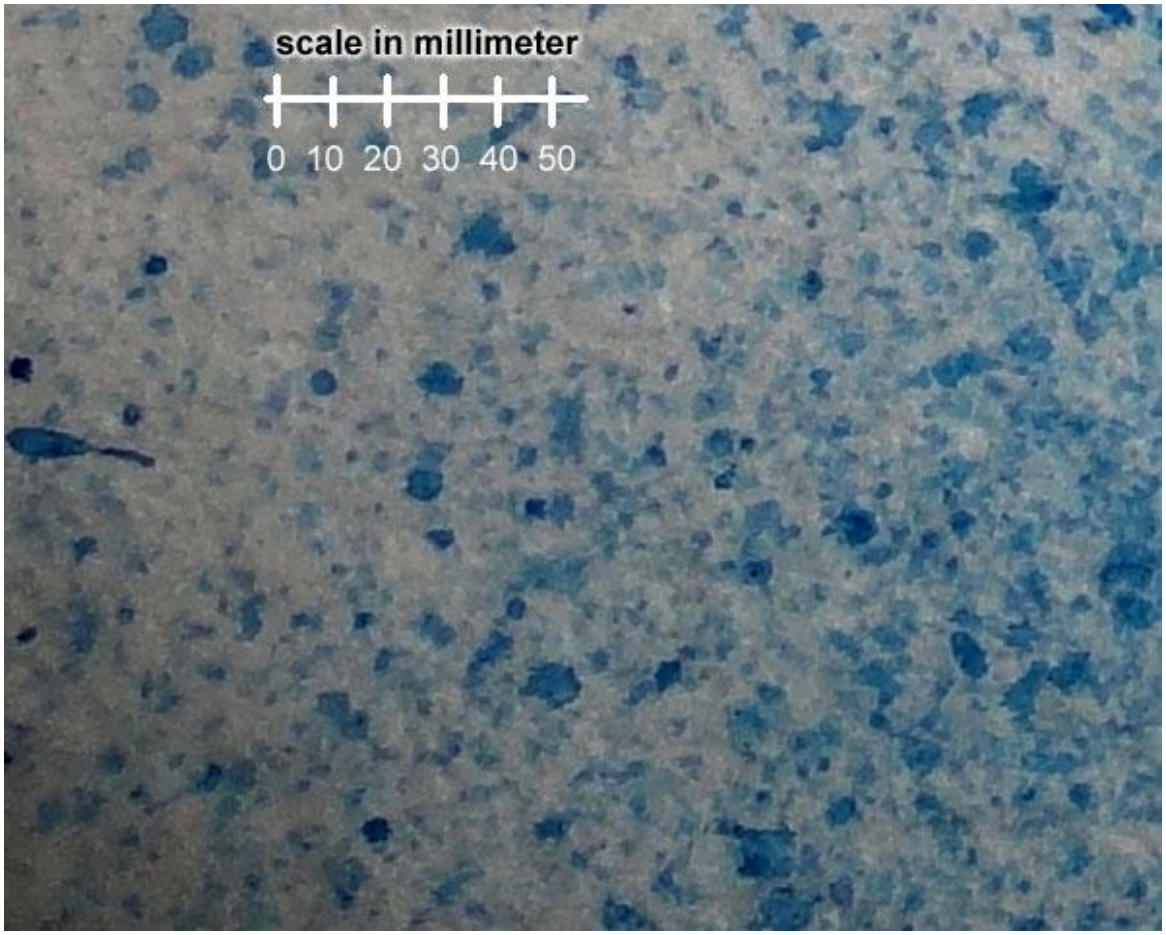


Figure9: 3x zoom of the significant result using Sony Cyber-shot

So, finally the cell samples were prepared by mixing cells suspended in DMEM, 10% DMSO, 20% EG and 1M sacrose. A drop of cell suspension was taken and dyed with trypan blue and was counted using haemocytometer like in section 3.5. The cell counted showed $1.8x10^7$cells/ml. the vitrification process was carried out using flow rate 0.5ml/min and N_2 gas pressure 0.8bar. After vetrification, the vitrified samples were stored in MVE 120L vapour phase LN2 vessel (to maintain -180 c temp) [Figure11] for 1 night and was thawed after a night. The thawing process is carried out by following the procedure in section 3.4, and the cells were counted as in section 3.5. Approximately, $1.3x10^7$cells/ml was viable, which is 72% viability (figure8).

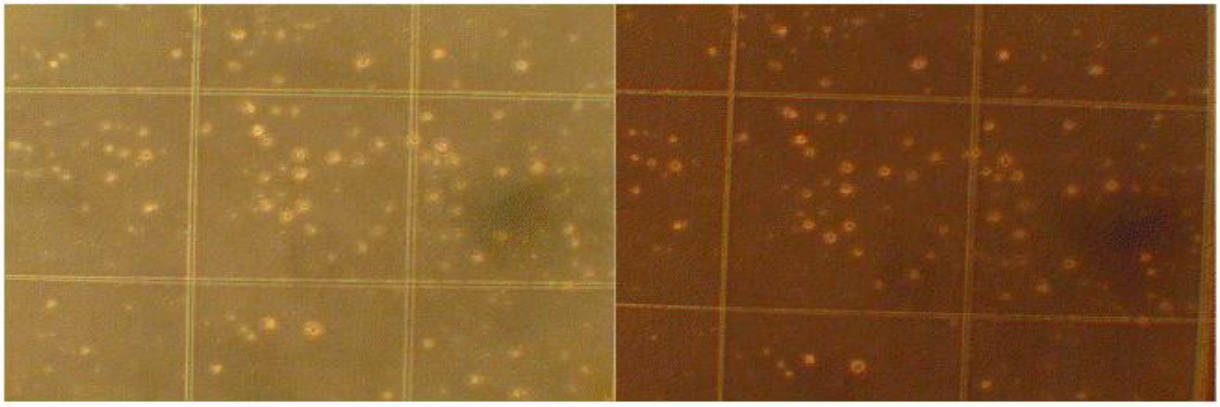


Figure10: Trypan blue assay using haemocytometer under carl zeiss phase contrast microscope.

Figure11: MVE 120L vapour phase LN2 vessel (to maintain -180 c temp)

4.3. Comparison

vitrification technique	Viability
plunging method	67%
dispersed droplets by N_2 gas	72%

Table3: comparison between the two vitrification methods.

From the result shown in Table3, we can conclude that viability can be increased by the new method.

5. CONCLUSION

In summary, an effective way of vetrification for the cryopreservation of MNCs using Nitrogen gas vapour to create small droplets is introduced. The N_2 gas also helped in precooling of the cells sample. And the most challenging part is that, it can be carried out in large scale in compare to the use of straw.

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